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Large Scale Synthesis of Recombinant Human Thyrotropin Using Methotrexate Amplification: Chromatographic, Immunological, and Biological Characterization*

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ABSTRACT

Studies of human TSH (hTSH) structure and function have been limited by difficulties in producing large quantities of recombinant hormone. We describe a system for the stable expression of high levels of recombinant human TSH (rec hTSH) using a mutant form of dihydrofolate reductase (dhfr) as an amplifiable dominant selectable marker. A vector expressing both the hTSH α-subunit and the mutant dhfr was cotransfected with a hTSH β-subunit expression vector into dhfr-deficient cells. Amplification of the transfected sequences by methotrexate selection, followed by cell culture in a hollow fiber perfusion system, yielded rec hTSH production as high as 100,000 μU/mL. Immunoradiometric assays using five different antibodies revealed no differences in the immunological activities of rec hTSH and pituitary hTSH. Bioactivity was measured in a novel TSH bioassay coupling the generation of cAMP by a transfected hTSH receptor to the cAMP-dependent regulation of a luciferase reporter gene. The ED_{50} for bovine TSH in this bioassay was 1.4 ng/mL (3.5×10^{-11} mol/L). The ratio of the ED_{50} values for rec hTSH and pituitary hTSH was 1.0:1.1 (P = NS), indicating that the two TSHs were of equivalent potency.

In conclusion, we have developed techniques for the high level production of rec hTSH that is immunologically and biologically equivalent to pituitary hTSH. The ability to produce large quantities of rec hTSH using standard laboratory techniques should facilitate future studies, such as the development of clinically useful TSH analogs. (*J Clin Endocrinol Metab* **81:** 1184–1188, 1996)

TSH, FSH, LH, and CG are members of a family of glycoprotein hormones comprised of noncovalently bound α- and β-subunits. The α-subunit is common to all four hormones, whereas the β-subunits are unique, conferring on each hormone its biologic specificity (1). Recombinant forms of these hormones are currently being evaluated for the diagnosis and treatment of many endocrine disorders, including infertility (2) and thyroid malignancies (3). There has also been much work using site-directed mutagenesis to create genetically altered forms of glycoprotein hormones (4–7). These hormone analogs can be used to study structure-function relationships and, as such, represent a first step in the development of potentially useful hormone agonists and antagonists. However, such studies require the ability to synthesize large amounts of recombinant hormone.

The large scale synthesis of recombinant human TSH (rec hTSH) for pharmaceutical use has been described previously (8). Smaller amounts of rec hTSH (ranging from 100–220 μU/mL) have also been produced in other laboratories (9), including our own (10). To date, however, nonproprietary protocols for the production of rec hTSH in quantities sufficient for detailed biological characterization have not been published. We describe a novel system for the stable expression of up to 100,000 μU/mL rec hTSH using a mutant form of dihydrofolate reductase (dhfr) as an amplifiable dominant selectable marker. The immunoactivity and bioactivity of this rec hTSH were equivalent to those of hTSH of pituitary origin. For bioactivity determinations, we report the first use of a hTSH receptor (hTSH-R) bioassay system in which TSH-stimulated cAMP generation is coupled to the cAMP-dependent regulation of a luciferase reporter gene.

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Materials and Methods

Materials

Enzymes, cell culture reagents, and luciferin were purchased from Sigma Chemical Co. (St. Louis, MO) and Life Technologies (Grand Island, NY). Pituitary hTSH (RP-1, lot LER-1952; 1.5 IU/mg) and bovine TSH were generously provided by the National Hormone and Pituitary Program. Commercially available plasmids used in these studies included pMAMneo (Clontech Laboratories, Palo Alto, CA), pGL2P (Promega Corp., Madison, WI), and pCEP4 (Invitrogen, San Diego, CA). The pHbA3-dhfr vector has been described previously (11, 12). Hollow fiber cell culture systems with a 10,000-dalton cut-off were purchased from

Amicon (Danvers, MA). hTSH was measured in two immunoradiometric assays (IRMAs): the three-antibody Nichols TSH isotopic assay (Nichols Diagnostics, San Juan Capistrano, CA) and a two-antibody assay from Diagnostics Products Corp. (Los Angeles, CA). cAMP levels were determined by RIA (Incstar, Stillwater, MN).

Cell lines and tissue culture medium

The dhfr-deficient Chinese hamster ovary (CHO) cell line, DG44, was cultured in DMEM containing antibiotics and 10% FCS (DMEM/FCS). For the selection of dhfr$^+$ clones, the medium was changed to hypoxanthine- and thymidine-deficient αMEM medium with 10% dialyzed FCS and antibiotics. Human embryonal kidney (HEK-293) cells were cultured in DMEM/FCS.

Production of rec hTSH

A complementary DNA (cDNA) coding for the α subunit of hTSH (13) was cloned into the *Hin*dIII site of the dual promoter expression vector pHβA3-dhfr to create the plasmid pHβA3-α. A full-length hTSH β-subunit cDNA was constructed from a minigene containing the second and third exons of the hTSHβ gene (14) by overlap-extension PCR and was confirmed by DNA sequencing. This hTSHβ cDNA was then cloned into the *Xho*I site of pMAMneo to create pMAMneo-β. The pHβA3-α and pMAMneo-β plasmids were cotransfected into DG44 cells by calcium phosphate-DNA coprecipitation and cultured in selection medium. Clonal cell lines secreting rec hTSH were then cultured in sequentially increasing concentrations of methotrexate (MTX) up to 10 μg/mL. After MTX amplification, the cell line producing the highest amount of rec hTSH was grown in a hollow fiber bioreactor according to the manufacturer's instructions. Medium containing secreted rec hTSH was collected from the extracellular space every 3–4 days.

Chromatographic and immunological analysis

Approximately 100,000 μU of both rec hTSH and pituitary hTSH were run on an 86-cm Sephadex G-75 column (Pharmacia, Piscataway, NJ). Fractions (2.0 mL) were collected at 0.18 mL/min in ammonium acetate (200 mmol/L; pH 6.9) at 4 C and assayed for hTSH by IRMA. Immunological comparisons of rec hTSH and pituitary hTSH were carried out by serially diluting each hTSH and comparing their immunoactivities in two hTSH IRMAs.

Measurement of TSH bioactivity

PCR was used to amplify a cAMP response element (CRE)-containing region of the human common glycoprotein hormone α-subunit gene from −180 to +44 bp (15, 16). Two copies of this PCR product were cloned in tandem upstream of the luciferase gene in the reporter plasmid pGL2P to give pGL2P-αCRE. A 2.5-kb hTSH-R cDNA (17, 18) was cloned into pCEP4 to create the expression vector pCEP4-hTSHR. For transfection, HEK-293 cells were harvested from two confluent 225-cm^2 flasks, washed three times with cold DMEM, and resuspended in 2.7 mL cold DMEM containing 30 μg plasmid. The cells were then divided equally into three cuvettes (4-mm path length) and electroporated using a BTX-600 set at 270 volts, 800 μF, and 72 ohms resistance, with resulting pulse lengths of 10.7–11.3 ms. After electroporation, cells were resuspended in 50 mL DMEM/FCS and grown overnight in sterile Falcon 2063 tubes (Falcon Plastics, Oxnard, CA; 1.0 mL/tube). For bioassay, transfected cells were treated overnight with TSH in assay medium [DMEM containing insulin (10 mg/L), transferrin (5.0 mg/L), BSA (10 g/L), 100 μmol/L $MgSO_4$, 100 μmol/L $ZnSO_4$, and 0.125 mmol/L isobutylmethylxanthine]. The cells were pelleted and washed with PBS, and cell lysates were analyzed for luciferase activity, as described previously (19). Dose-response curves as well as calculations of potency (ED_{50}) and 95% confidence intervals (95% CI) were determined from the program Allfit (20).

Results

Production of rec hTSH by stably transfected DG44 cells

The dhfr-deficient CHO cell line, DG44, was transfected with two vectors: pHβA3-α, expressing both the hTSH α-subunit and a mutant form of dhfr, and pMAMneo-β, expressing the hTSH β-subunit. Transfected cells were selected for the expression of exogenous dhfr by growth in hypoxanthine- and thymidine-deficient medium. Most of these dhfr-expressing clones would also be expected to express the TSH α-subunit cDNA present in pHβA3-α. Coexpression of hTSHβ by the pMAMneo-β vector in these hTSHα-producing cells resulted in the production of rec hTSH. As shown in Table 1, of the 80 initial dhfr$^+$ clones, 76% secreted at least 5 μU/mL rec hTSH, whereas 25% of the clonal lines produced more than 30 μU/mL rec hTSH. TSH-producing clones were then cultured in sequentially increasing concentrations of MTX, resulting in the amplification and overexpression of the transfected hTSH subunit cDNAs. Table 2 illustrates that sequential MTX amplification was associated with a greater than 40-fold increase in TSH production, with levels as high as 3300 μU/mL in monolayer cultures. These levels of TSH production were maintained for more than 3 months of continuous culture. The highest producing cell line was transferred to a hollow fiber perfusion cell culture system, where maximal TSH production plateaued at approximately 100,000 μU/mL.

Analysis of rec hTSH

Column chromatography (Fig. 1) demonstrated that rec hTSH had a higher apparent mol wt than the pituitary form. To determine whether this difference was associated with altered immunogenicity, we compared the immunoactivities of rec hTSH and pituitary hTSH. Serial dilutions of both forms of hTSH were measured in two different hTSH IRMAs: a two-antibody assay from Diagnostics Products Corp. and a three-antibody assay from Nichols Institute. As shown in Fig. 2, the immunological activities of the serially diluted TSHs were parallel to each other in both IRMAs. Thus, recombinant and pituitary hTSHs were recognized as immunologically equivalent molecules by the five antibodies used in these two assays.

Data from the parallel line assays were used to calculate equivalent amounts of rec hTSH and pituitary hTSH (based on immunoactivity) for measurements of bioactivity. For these studies, we developed a bioassay coupling TSH-stimulated cAMP generation to the transcriptional activation of a CRE-containing luciferase reporter gene. The plasmid pGL2P-αCRE (Fig. 3A) was created by cloning tandem CRE-containing regions of the common glycoprotein hormone α-subunit gene upstream of the luciferase reporter gene in pGL2P. We then expressed pGL2P-αCRE in HEK-293 cells and confirmed that the generation of cAMP by forskolin increased luciferase activity in a dose-dependent manner (Fig. 3B). Next, both pGL2P-αCRE and a plasmid expressing the hTSH-R (pCEP4-hTSHR) were cotransfected into HEK-

TABLE 1. TSH secretion by DG44 cells cotransfected with pHβA3-α and pMAMneo-β and selected for dhfr$^+$ phenotypes

TSH (μU/mL)	No. of TSH-producing clones (of 80 original dhfr$^+$ clones)
>5	61
>30	20
>100	4

TABLE 2. TSH secretion by DG44 cells after MTX amplification (10 μg/mL)

Clone	Pre-MTX amplication TSH (μU/mL)	Post-MTX amplification TSH (μU/mL)
1	31	290
2	108	947
3	85	3200
4	143	2260
5	81	3300
6	145	306

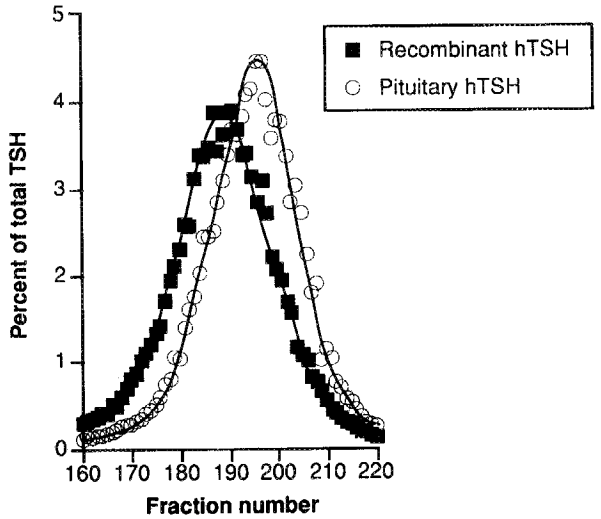

FIG. 1. Relative mol wt of recombinant and pituitary TSHs. Rec hTSH (■) and pituitary hTSH (○) were each run on the same 86-cm Sephadex G-75 column, and eluted fractions were assayed for TSH by IRMA. The hTSH content of each fraction was expressed as a percentage of the total amount of hTSH applied to the column. Recovery rates were 96.9% for rec hTSH and 99.5% for pituitary hTSH.

293 cells. As shown in Fig. 3C, treatment of these cells with TSH resulted in a dose-dependent increase in luciferase activity. Using highly purified bovine TSH (27 IU/mg), the ED_{50} of this bioassay was 1.4 ng/mL (95% CI, 0.2–8.3 ng/mL), equivalent to 38 μU/mL or 3.5×10^{-11} mol/L. When the bioactivities of rec hTSH and pituitary hTSH were compared using this assay (Fig. 4), the dose-response curves were superimposable. In three separate bioassays, with the ED_{50} for rec hTSH in each assay arbitrarily set at 1.0, the ED_{50} for pituitary hTSH was 1.10 (95% CI, 0.88–1.32; P = NS, by Mann-Whitney test). Thus, rec hTSH and pituitary hTSH were of equivalent potency in this homologous hTSH-R bioassay.

Discussion

The ability to produce recombinant glycoprotein hormones has greatly increased our understanding of their structure and function (21). Work on the gonadotropins has been particularly active, with approaches including site-directed mutagenesis (4), chimeric constructs (5), and isoform studies (22). Similar studies on TSH (23) have been limited by difficulties in producing the quantities of wild-type and mutant rec TSHs required for detailed characterization. Part of this difficulty appears to be due to the instability of the TSH β-subunit relative to the β-subunits of the other glycoprotein hormones (24). Commercial quantities of rec hTSH are being produced for pharmaceutical use (8); however, this report is the first description of a nonproprietary method for the large scale synthesis of rec hTSH.

We previously used the dual promoter expression vector pHβA3-dhfr to overexpress avian Ca^{2+} transport adenosine triphosphatase in fibroblasts (12). pHβA3-dhfr contains both a cDNA encoding a mutant form of the dhfr gene driven by a simian virus 40 promoter-enhancer as well as a multiple cloning site downstream of a β-actin promoter. In the presence of MTX, the mutant dhfr serves as a dominant selection marker in dhfr-deficient cells, resulting in the amplification and overexpression of genes within the multiple cloning site (11). We adapted this technique for the expression of the heterodimeric hTSH protein by cotransfecting a pHβA3-dhfr vector containing the hTSH α-subunit (pHβA3-α) with a vector expressing the hTSH β-subunit (pMAMneo-β). The efficiency of this process was illustrated by the 80 $dhfr^+$ clones generated on initial selection, 76% of which secreted rec hTSH. Subsequent stepwise selection with increasing concentrations of MTX resulted in cell populations stably expressing as much as 3300 μU/mL rec hTSH in monolayer culture and 100,000 μU/mL in hollow fiber culture. Watanabe and colleagues (9), using a wild-type dhfr expression vector, reported synthesizing up to 217 μU/mL rec hTSH after MTX amplification. The 15-fold higher levels of rec hTSH production (in monolayer) achieved in our system probably resulted from the greater degree of amplification associated with the mutant form of dhfr present in the pHβA3-dhfr expression vector (11).

Mol wt sizing by column chromatography indicated that rec hTSH had a higher apparent mol wt than the pituitary form. This finding was most likely due to differences in the length of hTSH β-subunit, the degree of hTSH glycosylation, or both. Although the sequence of the hTSH β-subunit cDNA predicts a length of 118 amino acids, the carboxy-terminal 6 amino acids are not present on the β-subunit of pituitary-derived hTSH (1). In contrast, the β-subunit of CHO cell-derived rec hTSH does not appear to undergo carboxy-terminal processing (25). Although this difference in length does not alter hTSH bioactivity (25), it may result in changes in apparent mol wt. The glycosylation of rec and pituitary hTSHs has been analyzed in a series of detailed studies by Weintraub and colleagues (23, 26, 27). When they determined the carbohydrate content of each TSH on a picomoles of sugar per micrograms of protein basis, rec hTSH synthesized in CHO cells contained 37% more carbohydrate than the pituitary form.

To determine whether this difference in mol wt had functional implications, we compared the immunogenicities and bioactivities of rec hTSH and pituitary hTSH. In immunological studies, rec hTSH and pituitary hTSH exhibited parallel serial dilution curves in two different IRMAs. These findings indicated that five different antibodies directed against both the α- and β-subunits of hTSH were unable to detect immunological differences between rec hTSH and pituitary hTSH. These findings were also consistent with previous studies reporting a high degree of immunological similarity between rec and pituitary hTSHs (26, 28). The bioactivities of rec hTSH and pituitary hTSH were compared

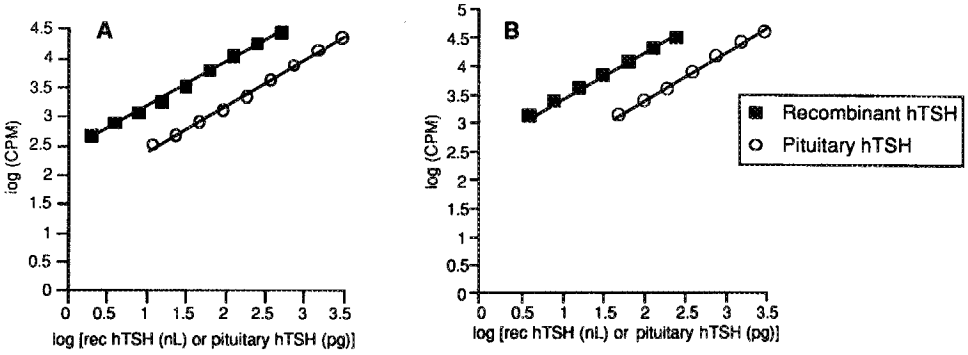

FIG. 2. Immunoreactivities of rec and pituitary TSHs. Rec hTSH (■) and pituitary hTSH (○) were assessed for parallel immunoactivity profiles after serial dilution using two immunoradiometric assays. A, Two-antibody assay from Diagnostic Products Corp. B, Three-antibody assay from Nichols Institute.

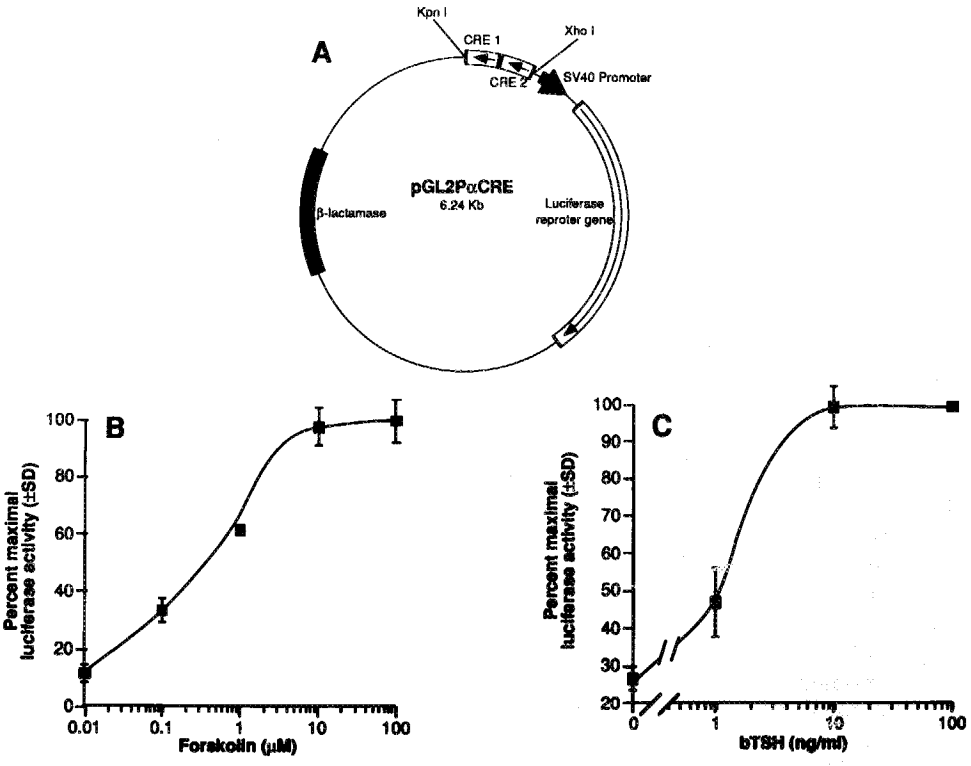

FIG. 3. TSH bioassay coupling generation of cAMP by a transfected hTSH-R to regulation of a CRE-containing luciferase reporter gene. TSH bioassays were performed in HEK-293 cells transfected with the pSVL-hTSHR and pGL2P-αCRE vectors as described in the text. A, The vector pGL2P-αCRE containing tandem CREs upstream of a luciferase reporter gene. B, Confirmation of cAMP-dependent luciferase activity using forskolin in cells transfected with pGL2P-αCRE. C, Dose-response curve for highly purified bovine TSH (bTSH). *Data points* are expressed as the mean ± SD of triplicate determinations, with dose-response curves calculated using Allfit.

in a homologous bioassay coupling hTSH-R-mediated increases in cAMP to the transcriptional activation of a CRE-containing luciferase reporter construct. In this bioassay system, equal amounts of pituitary and rec TSH (by immunoassay) exhibited comparable biological potencies. Thus, rec hTSH appeared to be both immunologically and biologically equivalent to the naturally occurring pituitary hormone. Other investigators also examined the bioactivities of rec and pituitary hTSHs. In a homologous bioassay system using human fetal thyroid cells, Huber and colleagues (29) found that immunologically equivalent amounts of rec hTSH and pituitary hTSH had similar bioactivities. Thotakura and colleagues (26) analyzed the bioactivities of rec and pituitary TSHs in heterologous bovine and rat bioassay systems. In contrast to our results and those of Huber, their studies suggested that rec hTSH was less bioactive than pituitary hTSH. However, these researchers proposed that this difference in bioactivity may have resulted in part from the use of heterologous bioassay systems. Thotakura and colleagues also found that the potency of their rec hTSH increased after the enzymatic removal of sialic acid residues. Thus, some differences in the bioactivities of different rec hTSH preparations may be due to variations in glycosylation.

The luciferase-coupled TSH bioassay described above enabled the sensitive, inexpensive, and nonradioactive detection of cAMP generation in cells expressing the hTSH-R. Similar assay systems have been reported for both FSH (16) and LH/CG (30). As shown in Fig. 3B, the amplification of cAMP signaling by luciferase enabled the detection of a TSH dose of 1.0 ng/mL (2.5×10^{-11} mol/L) with an ED_{50} of 1.4 ng/mL (3.5×10^{-11} mol/L). This technique could also be adapted to the measurement of other signal transduction systems by substituting the appropriate response elements for the CREs. For example, the use of phorbol response elements in the luciferase reporter construct may enable the measurement of TSH-stimulated protein kinase C pathway-dependent signaling.

The successful use of rec hTSH to detect functioning thy-

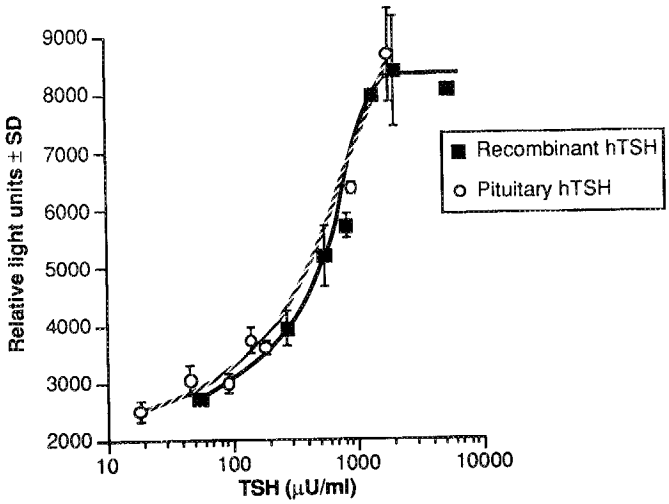

FIG. 4. Bioactivities of rec and pituitary TSHs in a luciferase-coupled hTSH-R bioassay system. Bioassays of rec hTSH (■) and pituitary hTSH (○) were performed as described in the text. Dose-response curves were calculated using the program Allfit. Rec hTSH: ED_{50}, 687 μU/mL; 95% CI, 467-1023 μU/mL. Pituitary hTSH: ED_{50}, 513 μU/mL; 95% CI, 323–812 μU/mL.

roid tissue in patients with thyroid cancer (3) holds great promise for the management of patients with thyroid malignancies. Further advances may be achieved with genetically altered forms of hTSH, such as superagonists to detect thyroid tissue, or antagonists to suppress the growth of residual malignant tissue. However, the development of such hTSH analogs will require systems capable of producing large amounts of wild-type and mutant forms of rec hTSH. In this report, we have described methodology that can be used to produce the quantities of rec hTSH required for these studies. We have also described a novel TSH bioassay coupling cAMP generation to luciferase expression. These systems combined with the use of techniques such as site-directed mutagenesis will improve our understanding of TSH action and may prove useful in the development of clinically testable TSH analogs.

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